

PERPUSTAKAAN UMP



0000086922

ENZYMATIC DEPOLYMERIZATION OF LIGNIN BY LACCASES

Nor Hanimah Hamidi

This is a thesis submitted to the University of Nottingham for the degree of Ph.D in the Faculty of Engineering

November 2013

6/19/11/14 PERPUSTAKAAN UNIVERSITI MALAYSIA PAHANG	
No. Perolehan 086922	No. Panggilan TS 933 .L5 H36 2013 Thesis
Tankh 01 JUL 2014	

Department of Chemical and Environmental Engineering
University of Nottingham
Nottingham
NG7 2RD
United Kingdom

ABSTRACT

More than half of platform petrochemicals are aromatic, whereas the only large-scale, naturally-occurring, renewable source of aromatics is lignin. Chemical depolymerization of lignin requires extreme conditions, and results in extensive destruction of the aromatic rings and/or char formation. By contrast, enzymatic lignin depolymerization occurs under mild conditions with retention of the aromatic nuclei. Therefore, laccase from *Agaricus bisporus* (LAB) and from *Trametes versicolor* (LTV) with the mediator, ABTS (2,2'-azino-bis(3 ethyl benzthiazoline-6-sulphonic acid)) were used to depolymerize lignin (sodium lignosulphonate) under mild reaction conditions with the aim to obtain high concentrations of value-added chemicals. The depolymerization in the presence of LTV was higher than LAB, which resulted from the high catalytic activity of LTV. Lignin degradation resulted in formation of complex product mixtures. Therefore the products were fractionated and analyzed by different analytical techniques including GPC (for preliminary screening), HPLC and GCMS (for product characterization and quantification), and NMR (for fingerprint analysis). Products included guaiacol, vanillin, acetovanillone, vanillic acid, homovanillyl alcohol, phenol, 4-methylbenzaldehyde, catechol, *p*-toluic acid, 4-hydroxybenzaldehyde, tyrosol, isovanillin, and 3-hydroxy-1-(4-hydroxy-3-methoxyphenyl) propan-1-one, and the total yield of monomers from lignin was 9.8 % in the presence of LTV. The parameters involved in the depolymerization process were optimized to increase the yield of monomers. The efficiency of laccase mediators was also explored by the use of 2,2,6,6-tetramethylpiperidin-1-yloxy (TEMPO), 1-hydroxybenzotriazole (HBT), *N*-hydroxyphthalimide (HPI) and violuric acid (VLA) in the depolymerization of sodium lignosulphonate. However, the catalytic depolymerization in the presence of these mediators was lower than ABTS. In order to improve the solubility of the substrate for the depolymerization process, screening of ionic liquids that are compatible with LAB was deployed in order to find laccase-friendly ionic liquids for further use in lignin depolymerization. The study has found [C₄mim] [L-tartrate] as the best ionic liquid tested, that increased the activity of LAB by 90 %. In conclusion, enzymatic depolymerization of lignin offers a greener process than the chemical methods, and also provides a more efficient method to obtain monomers of valuable specialty chemicals under mild reaction conditions.

TABLE OF CONTENTS

ACKNOWLEDGEMENT.....	i
DECLARATIONS	ii
ABSTRACT	iv
TABLE OF CONTENTS	v
LIST OF TABLE	xi
LIST OF FIGURES.....	xiii
LIST OF ABBREVIATIONS	xvii
Chapter 1	1
AIM AND SCOPE OF THE THESIS.....	1
Chapter 2	4
LITERATURE REVIEW.....	4
2.1 The Need for Lignin Utilization	4
2.2 Lignocellulose and lignin	5
2.2.1 Lignin Preparation.....	10
2.3 Lignin Depolymerization.....	14
2.4 Enzymatic Depolymerization of Lignin	15
2.4.1 Laccase.....	16
2.4.2 Lignin Peroxidase.....	23
2.4.3 Manganese Peroxidase	24
2.4.4 Versatile Peroxidase.....	26
2.5 Ionic Liquids.....	27
2.5.1 Ionic Liquids as Solvents for Lignin	30

2.5.2	Ionic Liquids as Solvents for Laccase.....	33
Chapter 3	35
	MATERIALS & METHODS.....	35
3.1	Materials	35
3.1.1	Buffer Preparation	35
3.1.2	Laccase	36
3.1.3	Lignin	36
3.2	Laccase Activity	36
3.2.1	Laccase from <i>Trametes versicolor</i> (LTV).....	36
3.2.2	Laccase from <i>Agaricus bisporus</i> (LAB)	37
3.3	Mediated Oxidation with Laccase	37
3.4	Lignin Derived Compounds as a Substrate	41
3.5	Analysis Strategy	41
3.5.1	Gel Permeation Chromatography.....	42
3.5.2	Nuclear Magnetic Resonance.....	43
3.5.3	Elemental Analysis.....	43
3.5.4	High Performance Liquid Chromatography with UV detector.....	44
3.5.5	Gas Chromatography Mass Spectroscopy	45
3.6	Ionic Liquids as Potential Solvents for Lignin Depolymerization.....	46
3.6.1	Ionic Liquids	46
3.6.2	Assays for Laccase from <i>Agaricus Bisporus</i> Activity in Ionic Liquids.....	47
3.6.3	Ionic Liquid Miscibility in Water.....	48
3.7	Determination of Michaelis-Menten Parameters.....	49
3.7.1	Enzyme kinetics by Michaelis-Menten and Lineweaver-Burke Plot.....	49
3.7.2	Enzyme kinetics via Non-linear Regression Analysis	51

Chapter 4	54
DEVELOPMENT OF ANALYTICAL METHODS AND THEIR USE IN PRELIMINARY TESTS OF LIGNIN DEPOLYMERIZATION USING LACCASE FROM <i>AGARICUS BISPORUS</i>	54
4.1 Introduction	54
4.2 Activity of Laccase from <i>Agaricus bisporus</i> (LAB)	54
4.2.1 The Effect of Temperature on the Activity of LAB	57
4.3 LAB catalyses the Oxidation of Sodium Lignosulphonate	59
4.4 Preliminary Screening of Fractions by GPC	63
4.5 Fingerprint Analysis of Different Fractions by ¹ H-NMR	65
4.5.1 The Effect of LAB Concentration	69
4.5.2 The Effect of ABTS Concentration on the Formation of Products	71
4.6 Elemental Analysis (EA)	74
4.7 GCMS Analysis	75
4.8 HPLC Analysis with UV detector	80
4.9 Effect of incubation time on Product Formation	82
4.10 Discussion	85
Chapter 5	87
LACCASE FROM <i>TRAMETES VERSICOLOR</i> AS A POTENTIAL ENZYME FOR DEPOLYMERIZATION OF SODIUM LIGNOSULPHONATE	87
5.1 Introduction	87
5.2 Laccase from <i>Trametes versicolor</i> (LTV) as a Potential Enzyme	87
5.3 Temperature Affects the Activity of LTV	89
5.4 Mediated oxidation of Sodium Lignosulphonate by LTV	90
5.4.1 Elemental Analysis of the Aqueous Fraction and the Solid Residue	98
5.4.2 GCMS analysis after Derivatization	99

5.5	Attempts to Quantify Products by HPLC with UV detector	104
5.5.1	Effect of Incubation Time on Product Formation at 60 °C.....	107
5.5.2	Effect of Temperature on Lignin Depolymerization.....	110
5.6	Effect of Incubation Time on Product Formation at 30 °C	110
5.7	Lignin Derived Compounds as a Substrate	112
5.7.1	The Oxidation of Vanillin	113
5.7.2	The Oxidation of Acetovanillone.....	116
5.7.3	The Oxidation of Guaiacol.....	119
5.7.4	The Oxidation of Vanillic Acid.....	121
5.7.5	The Oxidation of Homovanillyl Alcohol	124
5.8	Discussion.....	127
Chapter 6	130
TOWARDS UNDERSTANDING OF THE LACCASE-MEDIATOR SYSTEM ...		130
6.1	Introduction	130
6.2	Laccase Activity in the Presence of TEMPO and HBT	131
6.3	Mediation Efficiency towards Lignin Depolymerization.....	133
6.3.1	TEMPO	133
6.3.2	HBT.....	137
6.3.3	HPI	140
6.3.4	VLA.....	143
6.4	Discussion.....	146
Chapter 7	150
IONIC LIQUIDS AS POTENTIAL SOLVENTS FOR LIGNIN DEPOLYMERIZATION		150
7.1	Introduction	150

7.2	The Activity of ABTS in the Presence and Absence of [C ₄ eim] [C ₂ SO ₄]	152
7.3	Effect of [C ₄ mim] [lactate] Concentration on LAB activity	154
7.4	Screening of Ionic Liquids	155
7.4.1	Effect of Imidazolium Based Ionic Liquids on LAB Activity	156
7.4.2	Effect of Quaternary Ammonium Based Ionic Liquid on LAB Activity	160
7.4.3	Effect of Phosphonium Ionic Liquids on LAB Activity	164
7.4.4	Effect of Pyridinium Ionic Liquids on LAB Activity	166
7.4.5	Effect of Piperidinium and Pyrrolidinium Ionic Liquid on LAB Activity 167	
7.5	Discussion.....	168
Chapter 8	172
	DISCUSSION AND CONCLUDING REMARKS.....	172
8.1	Summary of Results	172
8.2	Improvement of the Process	176
8.3	Improvement of Analytical Methods.....	181
8.4	Economic Considerations	183
8.4.1	Production of High Value Chemicals	185
8.5	Consideration for a Large Scale Depolymerization Process	187
8.6	Concluding Remarks	189
9.	REFERENCES.....	191
	APPENDICES.....	207
	Appendix A.1 GCMS Analysis of Lignin Depolymerization Products (LAB)	207
	Appendix A.2 GCMS Analysis of Lignin Depolymerization Products (LTV).....	210
	Appendix A.3 GCMS Analysis of Lignin Depolymerization Products after Derivatization (LTV).....	216
	A.3.2 GCMS standard calibration curve	223

Appendix A.4 HPLC Analysis of Lignin Depolymerization Products	225
A.4.1 HPLC authentic standard peak area	225
A.4.2 HPLC standard calibration curves.....	227
Appendix A.5 Lignin Derived Compounds as a Substrate.....	228
A.5.1 The Oxidation of Vanillin (2)	228
A.5.2 The Oxidation of Acetovanillone (3)	230
A.5.3 The Oxidation of Guaiacol (1)	232
A.5.4 The Oxidation of Vanillic Acid (5)	233
A.5.5 The Oxidation of Homovanillyl Alcohol (4).....	236
Appendix A.6 Laccase Mediator System	238
A.6.1 Chromatogram of Aqueous Ethyl Acetate Extract Fraction of the LTV- catalyzed reaction Mediated by TEMPO	238
A.6.2 Chromatogram of Aqueous Ethyl Acetate Extract Fraction of the LTV- catalyzed reaction Mediated by HBT	240
A.6.3 Chromatogram of Aqueous Ethyl Acetate Extract Fraction of the LTV- catalyzed reaction Mediated by HPI	242
A.6.4 Chromatogram of Aqueous Ethyl Acetate Extract Fraction of the LTV- catalyzed reaction Mediated by VLA.....	244
Appendix A.7 List of ionic liquids used in this study	247
A.7.1 Imidazolium Based Ionic Liquid.....	247
A.7.2 Quaternary Ammonium Based Ionic Liquid	250
A.7.3 Phosphonium Based Ionic Liquid	253
A.7.4 Pyridinium based Ionic Liquid.....	255
A.7.5 Piperidinium and Pyrrolidinium based Ionic Liquid.....	256

LIST OF TABLE

Table 2.1 Comparison of reactions and prices of ligninolytic enzymes.....	27
Table 3.1 Sample preparation for elementary analysis.....	44
Table 4.1 The mass balance of product fractions (LAB).	61
Table 4.2 Elemental composition (LAB).	75
Table 4.3 Identification of products formed after liquid-liquid extraction (LAB).....	78
Table 4.4 The identities of the compounds in the aqueous ethyl acetate extract.	82
Table 5.1 Kinetic parameters for the oxidation of ABTS by different laccases.....	89
Table 5.2 The mass balance of product fractions (LTV).....	92
Table 5.3 Identification of products formed after liquid-liquid extraction (LTV).	94
Table 5.4 The comparison of products formed between LAB and LTV.....	96
Table 5.5 Elemental composition (LTV).....	98
Table 5.6 Identification of products formed (derivatization)	101
Table 5.7 Comparison of products concentration between GCMS and HPLC.....	107
Table 5.8 Products formed in the conversion of compounds representative of lignin catalyzed by LTV in the presence of ABTS.	114
Table 5.9 The percentage of unconverted reactant and undetected product.	115
Table 6.1 Effect of different substrate on the oxidation by LTV.	133
Table 6.2 Identification of products formed after the depolymerization of sodium lignosulphonate by LTV and mediated by TEMPO.....	136
Table 6.3 Identification of products formed after the depolymerization of sodium lignosulphonate by LTV and mediated by HBT.	138
Table 6.4 Identification of products formed after the depolymerization of sodium lignosulphonate by LTV and mediated by HPI.....	141
Table 6.6 Comparison of the products formed after enzymatic treatment of sodium lignosulphonate in the presence of ABTS, TEMPO, HBT, HPI and VLA.....	147
Table 6.7 Current market price for selected mediators	148
Table 7.1 Activity of LAB in the presence of imidazolium based ionic liquids and halides anion.....	156

LIST OF FIGURES

Figure 2.1 Oil and gas production profiles around the globe in 2008	5
Figure 2.2 Components of lignocellulosic biomass.	6
Figure 2.3 Woody plant.....	7
Figure 2.4 Lignin precursors.	8
Figure 2.5 Representation of softwood lignin polymer.....	9
Figure 2.6 Representation of isolated liginosulphonate polymer	11
Figure 2.7 Representation of an isolated Kraft lignin.	12
Figure 2.8 Schematic diagram of the lignin degradation steps.	16
Figure 2.9 Reduction of dioxygen (O_2) to water (H_2O) by laccase.....	17
Figure 2.10 Laccase active site.....	17
Figure 2.11 Schematic representation of laccase catalyzed redox cycles	18
Figure 2.12 Two types of mushrooms.....	19
Figure 2.13 Structures of some laccase mediators	20
Figure 2.14 Schematic representation of laccase catalyzed redox cycles for lignin oxidation in the presence of a mediator	21
Figure 2.15 Oxidation of ABTS in the presence of laccase	22
Figure 2.16 Schematic representation of lignin peroxidase (LiP) catalyzed redox cycles for veratryl alcohol (VA) oxidation	24
Figure 2.17 Schematic representation of manganese peroxidase (MnP) catalyzed redox cycles for Mn^{2+}	25
Figure 2.18 Schematic representation of versatile peroxidase (VP) catalyzed redox cycles for Mn^{2+}	26
Figure 2.19 Some commonly used ionic liquid systems	29
Figure 2.20 SEM images.....	31
Figure 2.21 The depolymerization of lignin to smaller lignin subunits.	32
Figure 3.1 Scheme for the fractionation method of lignin depolymerization products. .	39
Figure 3.2 Summary of the analysis strategy	42
Figure 3.4 Single phase and biphasic system of ionic liquids and water mixture.....	48
Figure 3.5 Determination of Michaelis-Menten parameters.	50

Figure 4.1 The oxidation of ABTS by LAB.....	55
Figure 4.2 Effect of ABTS concentration on the oxidation by LAB..	57
Figure 4.3 Effect of temperature on LAB activity..	58
Figure 4.4 The colour intensity of lignin product fractions..	60
Figure 4.5 Depolymerization of sodium lignosulphonate by LAB (analyzed by GPC). 64	
Figure 4.6 The ¹ H-NMR spectrum of aqueous ethyl acetate extracts.	66
Figure 4.7 ¹ H-NMR spectra for comparison of functional groups.....	68
Figure 4.8 The ¹ H-NMR spectra after treatment of sodium lignosulphonate with different LAB concentrations.....	70
Figure 4.9 Comparison between ABTS intensity before and after the treatment with LAB.....	72
Figure 4.11 Chemical structures of lignin depolymerization products (LAB).	76
Figure 4.12 The GCMS chromatograms of products (LAB)..	77
Figure 4.13 Solubility of dried aqueous fraction in different solvent	80
Figure 4.14 HPLC chromatograms of four fractions after LAB treatment.	81
Figure 4.15 Effect of incubation time on the production of chemicals	84
Figure 5.1 Effect of ABTS concentration on reaction rate of different laccase.....	88
Figure 5.2 The effect of temperature on LTV activity.	90
Figure 5.3 Chemical structures of lignin depolymerization products (LTV).....	95
Figure 5.4 The GCMS chromatograms of products (LTV).....	97
Figure 5.5 The GCMS chromatograms of products that have been extracted in ethyl acetate after derivatization.	100
Figure 5.6 Chemical structures of the lignin depolymerization (after derivatization) ..	102
Figure 5.7 The production of aliphatic compounds from lignin.	104
Figure 5.8 HPLC chromatograms of the products formed(LTV)	105
Figure 5.9 Concentration of lignin depolymerization products formed at 60 °C.....	106
Figure 5.10 Effect of incubation time on products formation	108
Figure 5.11 Thermal stability of LTV at 30 to 60°C.....	109
Figure 5.12 Comparison of lignin depolymerization products formed at 60 °C and 30 °C.....	110
Figure 5.13 The effect of incubation time on the product yield.....	111

Figure 5.14 Oxidation of vanillin.....	113
Figure 5.15 The ¹ H-NMR spectrum of the products formed after enzymatic treatment of vanillin by LTV.....	116
Figure 5.16 Oxidation of acetovanillone.....	117
Figure 5.17 The ¹ H-NMR spectrum of the products formed after enzymatic treatment of acetovanillone by LTV.....	118
Figure 5.18 Oxidation of guaiacol.....	119
Figure 5.19 Conversion of guaiacol by LTV in the presence of ABTS	120
Figure 5.20 The ¹ H-NMR spectrum of the products formed after enzymatic treatment of guaiacol by LTV.....	121
Figure 5.21 The conversion of vanillic acid	122
Figure 5.22 The ¹ H-NMR spectrum of the products formed after enzymatic treatment of vanillic acid by LTV.	123
Figure 5.23 Conversion of homovanillyl alcohol by LTV in the presence of ABTS. ..	125
Figure 5.24 The conversion of homovanillyl alcohol	125
Figure 5.25 The ¹ H-NMR spectrum of the products formed after enzymatic treatment of homovanillyl alcohol by LTV.....	126
Figure 6.1 The oxidation of <i>p</i> -anisilic alcohol by laccase from <i>Trametes villosa</i>	131
Figure 6.2 Synthetic mediators used in this study.....	131
Figure 6.3 Comparison of the rate of oxidation between ABTS, TEMPO and HBT by LTV.	132
Figure 6.4 Mechanism of TEMPO oxidation by laccase	134
Figure 6.5 The effect of incubation time on the depolymerization of sodium lignosulphonate mediated by TEMPO.	135
Figure 6.6 Mechanism of HBT oxidation by laccase	137
Figure 6.7 The effect of incubation time on the depolymerization of sodium lignosulphonate mediated by HBT.....	139
Figure 6.8 Mechanism of HPI oxidation by laccase	140
Figure 6.9 The chemical structure of 1,2-benzenedicarboxylic acid (HPI-P1) and 2-cyanobenzoic acid (HPI-P2).....	142

Figure 6.10 The effect of incubation time on the depolymerization of sodium lignosulphonate mediated by HPI.	142
Figure 6.11 Mechanism of VLA oxidation by laccase	144
Figure 6.12 The effect of incubation time on the depolymerization of sodium lignosulphonate mediated by VLA.	145
Figure 7.1 Cations and anions used in this study.	151
Figure 7.2 The absorbance changes during the oxidation of ABTS by LAB.	152
Figure 7.3 Time courses for ABTS oxidation in ionic liquid	153
Figure 7.4 Effect of [C ₄ mim] [lactate] concentration on the oxidation of ABTS	155
Figure 8.1 Comparison of the products formed after enzymatic treatment of sodium lignosulphonate in the presence of LAB and LTV (with different mediator, namely ABTS, TEMPO, HBT, HPI and VLA)	175
Figure 8.2 Schematic flow diagram of the activity of glucose: quinone oxidoreductase	176
Figure 8.3 Evolution of LTV activity.....	177
Figure 8.4 Redox catalysis of veratryl alcohol and ABTS	178
Figure 8.5 A partial view of the structure of suberin.	180
Figure 8.6 Thioacidolysis method.	182
Figure 8.7 The market value of lignin and its potential products	184
Figure 8.8 Potential lignin applications.....	185
Figure 8.9 Process flow sheet.....	187

LIST OF ABBREVIATIONS

Time, length, weight, volume and concentration:

h	hours
min	minutes
s	seconds
n	nanometres
µm	micrometres
g	gram
mg	milligram
µl	microliters
ml	milliliters
L	litres
mM	milimolar
M	molar

General abbreviations:

A	Absorbance
ACN	Acetonitrile
ABTS	2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)
BSTFA	bis(trimethylsilyl)trifluoroacetamide
°C	degree celcius
DCM	dichloromethane
DMSO-d ₆	deutrated dimethyl sulfoxide
EA	elementary analysis
EI	electron ionization
ET	electron transfer
EPR	electron paramagnetic resonance
GC	gas chromatography
GCMS	gas chromatography mass spectroscopy
GPC	gel permeation chromatograpy

HAT	hydrogen atom transfer
HBT	hydroxybenzotriazole
HPI	<i>N</i> -hydroxyphthalimide
HPLC	high performance liquid chromatography
K_m	value of substrate concentration at $1/2 V_{max}$
L	light path length
LiP	lignin peroxidase
LAB	laccase from <i>Agaricus bisporus</i>
LTV	laccase from <i>Trametes versicolor</i>
LMS	laccase mediator system
mV	millivolts
MnP	manganese peroxidase
MeOH	methanol
mol	moles
NMR	nuclear magnetic resonance
OD	optical density
ppm	part per million
[P]	product concentration
rpm	round per minute
RI	refractive index
SD	standard deviation
SEC	size exclusion chromatography
SEM	scanning electron microscope
[S]	substrate concentration
[S ₀]	initial substrate concentration
t	time
THF	tetrahydrofuran
TMCS	trimethylchlorosilane
TEMPO	2,2,6,6-tetramethylpiperidin-1-yloxy
uv	ultra violet
v_o	initial rate of reaction

V	rate of reaction
VOC	volatile organic compound
VLA	violuric acid
V_{max}	maximum velocity
v/v	volume per volume
w/w	weight per weight
w/v	weight per volume
λ	wavelength
ϵ	extinction coefficient

Chapter 1

AIM AND SCOPE OF THE THESIS

The aim and scope of the thesis is to explore the depolymerization of sodium lignosulphonate to value-added chemicals by an enzymatic process. In this study, commercial laccase was used since the isolation of laccase from lignin-degrading microorganisms such as white rot fungi are generally slow growing and may be difficult to cultivate at scale. In addition, laccase is produced on a large scale due to the widespread applications in biotechnology including in paper manufacturing, detergent formulations, bioremediation, biotransformation, lignocellulose processing, *etc.* (Yaver *et al.*, 2001). Therefore, a commercially available laccase was preferred. There are several factors that contribute towards the efficiency of the enzymatic conversion of lignin by laccase, which is complex from the chemical and biological points of view. A process was developed to study the effect of laccase from *Agaricus bisporus* (LAB) on the degradation of sodium lignosulphonate in the presence of 2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) as a mediator. After the enzymatic depolymerization by laccase, a complex mixture of the products was formed, that is extremely difficult to analyze. Therefore, a fractionation method was implemented to simplify the analysis process and a combination of analytical methods was deployed to identify the products.

Several studies have implemented size exclusion chromatography (SEC) to study the molecular weight distribution of the products (Majcherczyk and Huttermann, 1997; Nugroho *et al.*, 2010; Shleev *et al.*, 2006). Gel permeation chromatography (GPC) is a type of SEC that separates on the basis of size. Bourbonnais *et al.* (1995) have

demonstrated the use of GPC to analyze the oxidation of Kraft lignin by laccase from *Trametes versicolor*. From their observation, the process produced molecular weight averages between 7800 to 10500 gmol⁻¹ after several days of treatment. In this project, the aim would be to produce compounds that have a molecular weight below 1000 gmol⁻¹. Therefore, GPC was adopted as a part of the preliminary screening of the product distribution after fractionation.

Other than GPC, proton nuclear magnetic resonance (¹H-NMR) was implemented to provide chemical information about the products. In ¹H-NMR, a chemical shift is associated with the occurrence of various types of chemical resonance present in the sample. Therefore, this technique was used as a fingerprint analysis of the products. NMR analysis has become one of the important milestones for lignin chemistry. However, it has to be noted that the characterization of lignin depolymerization products is difficult due to the complex mixture of products and overlapping signals.

Therefore, gas chromatography mass spectroscopy (GCMS) was also employed to characterize the products. Pecina *et al.* (1986) demonstrated the use of GCMS for the analysis of lignin degradation products. In their work, a method of derivatization was implemented to increase the volatility as well as the detectability of the products. However, derivatization is not always necessary for GCMS unless the compound of interest cannot be detected. In addition, quantification by GCMS was carried out by measuring the peak area of individual components and comparing with authentic standards. Besides GCMS, high performance liquid chromatography (HPLC) has been used for quantification in several studies (Pecina *et al.*, 1986; Bourbonnais and Paice, 1990; Bourbonnais *et al.*, 1997; Vigneault *et al.*, 2007). Thus, an attempt was made to develop an analytical method by using reversed-phase high performance liquid chromatography (RP-HPLC) for the quantification of lignin depolymerization products in conjunction with GCMS analysis. In this study, the identification of the products by GCMS revealed five compounds formed after the enzymatic depolymerization by LAB. However, the yield was only 7.8 % of the total lignin used.

Therefore, the next aim was to further increase the product yield by using laccase from a different source, to influence the efficiency of product formation from the breakdown of sodium lignosulphonate. Therefore, laccase from *Trametes versicolor* (LTV) was studied. The optimum reaction condition in the presence of LTV was explored, with respect to the reaction time, temperature and also the stability of LTV during the course of the reaction.

Even though ABTS is known as the best mediator for laccase (Morozova *et al.*, 2007; Bourbonnais and Paice, 1992), there are more than 100 possible mediators which have been classified into two types, namely natural and synthetic mediators (Canas and Camarero, 2010). Since the synthetic mediators have been proven to be the most effective mediators by several authors (d'Acunzo *et al.*, 2002; Fabbrini *et al.*, 2002), a study on the effect of five synthetic mediators on lignin depolymerization was implemented. Despite the addition of mediators into the reaction however, there is a major drawback since they are expensive (Li *et al.*, 1999; Couto *et al.*, 2005). Therefore, the process for lignin depolymerization by LTV was designed to use the least amount of mediator as possible.

Laccase has a variety of applications. In some cases however, the processes are inefficient because the substrate is insoluble in water. Therefore, it would be desirable to identify enzyme-friendly solvents that can be used to solubilize the substrates. Ionic liquids are a relatively new type of non-aqueous solvent which often perform better in biocatalytic processes than conventional solvents (Cull *et al.*, 2000). Most importantly, there are millions of ionic liquids, offering a variety of chemical and physical properties. This allows the structure of the ionic liquids to be fine tuned to match the specific requirements of the desired process. Therefore, 106 ionic liquids were tested for their effect on laccase from *Agaricus bisporus* (LAB) using a new high throughput screening method (Rehmann *et al.*, 2012). 2,2'-Azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) was used as the substrate, and the Michaelis-Menten kinetic parameters were determined.

Chapter 2

LITERATURE REVIEW

2.1 The Need for Lignin Utilization

Petroleum feedstocks are used in industry to produce a variety of products including fine chemicals, *etc.* (Bender, 2000; Demirbas, 2005). As reported by the Association for the Study of Peak Oil and gas (ASPO), the production of petroleum will decline gradually every year starting from 2010 (Fig. 2.1). The decreasing supply of this feedstock has forced the need to find new alternatives to meet the high demands of value-added chemicals in various applications.

In a sense, the fossil fuels are a one-time gift that lifted us up from subsistence agriculture and eventually should lead us to a future based on renewable resources -
Kenneth Deffeyes (2001).

Therefore, an alternative approach was explored based on the potential of lignin as a renewable feedstock for the production of valuable aromatic chemicals that are usually derived from petroleum. According to Gargulak and Lebo (2000), there is an estimated 50 million tonnes of lignin available per year from pulping processes worldwide and only 2 % is in use for commercial applications (Gargulak and Lebo, 2000).

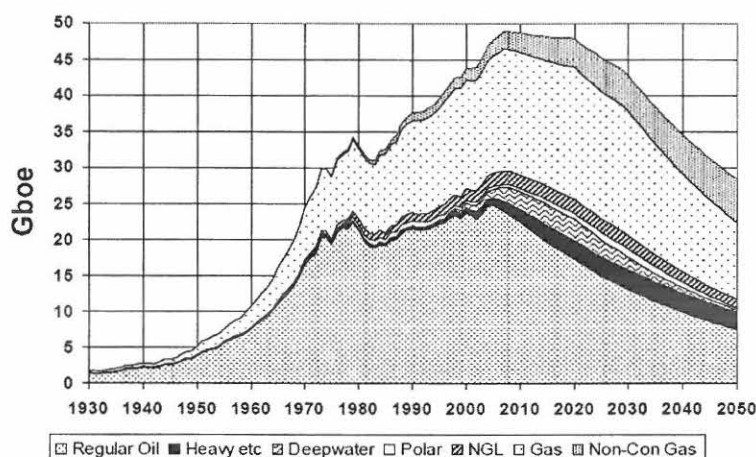


Figure 2.1 Oil and gas production profiles around the globe in 2008 taken from ASPO newsletter No. 100, April 2009. Gboe represents a giga barrel oil equivalent (ASPO, 2009).

2.2 Lignocellulose and lignin

The past decade has seen the rapid development of lignocellulosic biomass as a sustainable source of sugars for biotransformation into biofuels and valuable chemicals (Li *et al.*, 2008; Himmel *et al.*, 2007) especially in the fibre, paper, membrane, polymer and paint industries (Swatloski *et al.*, 2002). Lignocellulosic materials consist mainly of complex structures of the carbohydrates, cellulose (35-50%) and hemicelluloses (20 – 35 %), and lignin (5 - 30 %), a polyphenolic structure (Lnyd *et al.*, 2002; Zavrel *et al.*, 2009; Fig. 2.2).

Cellulose and hemicelluloses are easy to hydrolyze to their subunits (*e.g.* glucose, fructose, galactose, mannose, xylose). The transformations of celluloses and hemicelluloses to the monomer units are a relatively simple process. Numerous studies have attempted to obtain the conversion of cellulose to other products such as bioethanol as a promising alternative energy source to replace crude oil that is likely to suffer limited availability (Demirbas, 2005; Sun and Cheng, 2002). In 2009, Buckeye Technologies, Inc in association with Myriant and University of Florida have announced the development of a new generation bioethanol plant from cellulose which was believed

to be a step forward towards new source of fuel from renewable feedstock (Buckeye Technologies Inc., 2009).

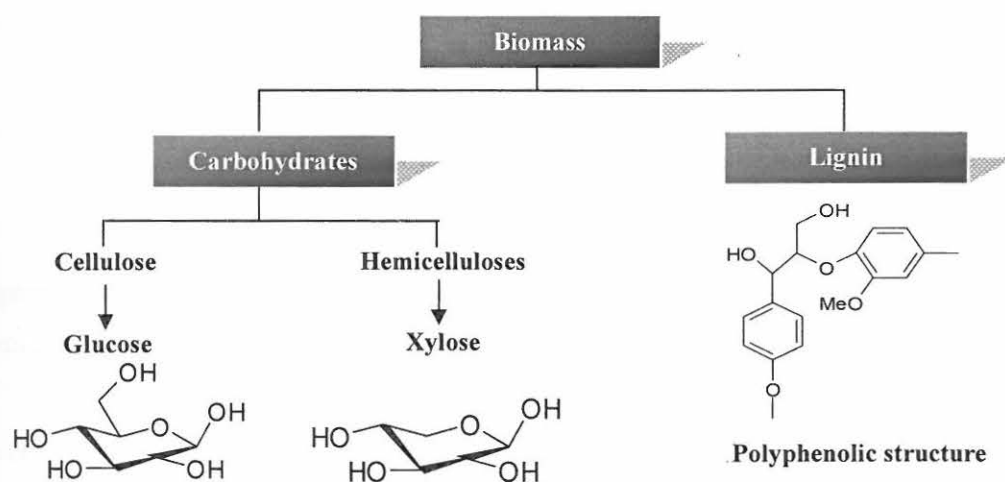


Figure 2.2 Components of lignocellulosic biomass taken from Rogers *et al.* (2002).

On the other hand, lignin is a polyphenolic material composed of phenylpropane units (Rogers *et al.*, 2002). Lignin is practically impossible to dissolve in water in its native form due to the irregular three dimensional cross-linked networks that bind the whole wood structure together to make a strong and resistant plant wood (Kilpelainen *et al.*, 2007). It may also play an important role in defence against pathogen attack and mechanical wounding (Hawkins *et al.*, 1997). The toughness of a plant depends on the percentage of lignin in the cell wall structure. For example, hardwood plants (Fig. 2.3a) contain more lignin compared to softwood plants (Fig. 2.3b) (Antai and Crawford, 1981).

The first serious discussion and analyses of lignin emerged in 1838 in a study by Anselme Payen (Frenh, 2000). He treated wood with nitric acid to remove part of the wood substances and left behind fibrous materials which he called 'cellulose'. He realized that the part that had been removed from the wood materials was rich in carbon content compared to the cellulose. He called the carbon-rich substance as an 'encrusting material' (French, 2000).

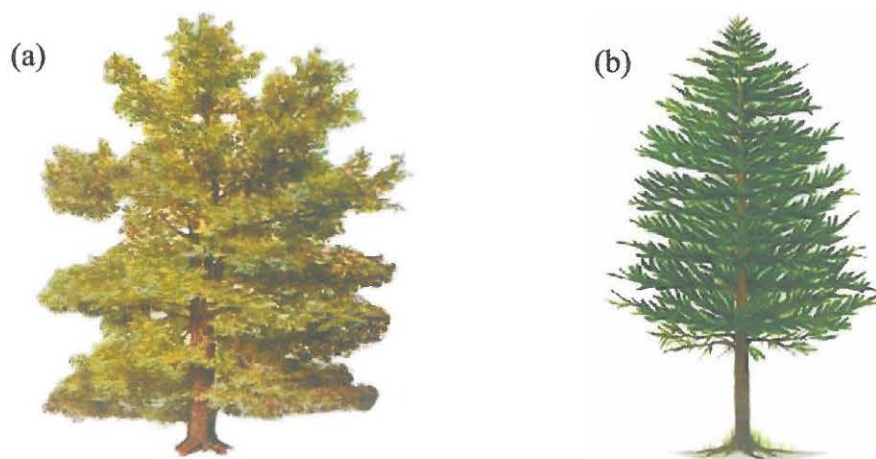


Figure 2.3 Woody plant (a): Hardwood plant (beech tree); (b): Softwood plant (pine tree) (taken from Karen whimsy, 2013 and Peacock river ranch, 2012)

Over the past 100 years, research into lignin has developed and enlarged beginning with work by Schulze in 1865 who first introduced the term ‘lignin’ (Lu and Ralph, 2010). Three years later, Erdmann in 1868 concluded that the non-cellulosic constituent in wood substances was aromatic. Further investigation of lignin was then demonstrated by Benedikt and Bamberger in 1890 in which they found that methoxyl groups were present in wood tissue but such tissues were lacking in cellulose materials (Brunow, 2001). Further research was done by Klason who came up with the idea in 1897 that lignin was chemically related to coniferyl alcohol (Sjöström, 1993).

Lignin is the second most abundant polymer in nature after cellulose (Annele, 1994; Leonowicz *et al.*, 1999; Li *et al.*, 2008; Zavrel *et al.*, 2009; Kilpelainen *et al.*, 2007; Adler, 1977). It is classified into three major groups which are softwood lignin, hardwood lignin and grass lignin based on the chemical structure of the monomer units (Adler, 1977) which build to form an aromatic, 3-dimensional and amorphous structure (Brown, 1985). Lignin is built from three precursors which are *p*-coumaryl alcohol, coniferyl alcohol and sinapyl alcohol as shown in Figure 2.4 and these precursors are incorporated in lignin as *p*-hydroxyphenyl (H), guaiacyl (G) and syringyl (S), respectively (Grabber *et al.*, 1997).

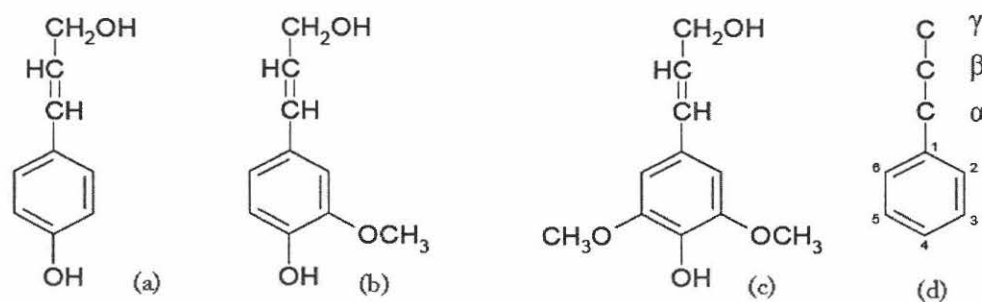


Figure 2.4 Lignin precursors (a) *p*-coumaryl alcohol (**H**); (b) coniferyl alcohol (**G**); (c) sinapyl alcohol (**S**); (d) model for numeration of a carbon skeleton which consist of the aromatic nucleus and 3-carbon side chain represented by γ , β and α (taken from Buswell and Odier, 1987).

These precursors then form different types of subunits of lignin macromolecules where the most abundant subunit is the guaiacylglycerol- β -aryl ether (β -O-4) substructure (40-60 %) followed by biphenyl and dibenzodioxocin, 5-5 (18 – 25 %), phenylcoumaran, β -5 (9 – 12 %), 1,2-diaryl propane, β -1 (7 – 10 %), phenylpropane α -aryl ether, α -O-4 (6 – 8 %), diaryl ether, 4-O-5 (4 -8 %) and β - β linked structures (Adler, 1977; Higuchi, 1990; Sakakibara, 1983; Fig. 2.5) *etc.* A large and growing body of literature has shown that there are no single repeating bonds between the subunits, but a random distribution of at least ten types of bonds (Argyropoulos and Menachen, 1997). The β -aryl ether (β -O-4) bond was the most common bond found in lignin molecule as shown in Fig. 2.5 (Buswell and Odier, 1987). The bonds in lignin are complicated and non hydrolysable, and are much more difficult to break down compared to cellulose and hemicelluloses that are just made from a simple structures and linked with β -1, 4-glucosidic bonds (Kuhad *et al.*, 1997).

Lignin has a high molecular weight which makes it a tough structure and prevents its uptake into the microbial cells (Eriksson *et al.*, 1990). Due to this fact, biological degradation of native lignin must occur through the activity of extracellular enzymes (Adler, 1977; Argyropoulos and Menachen, 1997; Kuhad *et al.*, 1997; Eriksson *et al.*, 1990) from lignin degrading microorganisms such as white rot fungi (Hatakka, 1994; Leonowicz *et al.*, 1999). White rot fungi have a unique ability to produce ligninolytic enzymes to degrade lignin. Wood-rotting fungi are divided into three groups which are